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Possibilities of Glutamate-Mediated Synaptic Like Activity In Somatosensory Mechanoreceptors Such As Pacinian Corpuscles?

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Abstract

Pacinian Corpuscles (PCs) are somatosensory mechanoreceptors, composed of a central neurite, surrounded by layers of lamellae that make up the inner core, which are specialized glial cells, and fibroblast derived outer-core lamellae of the surrounding capsule. Several components specific to a chemical synapse (Vglut1, Vamp1, SNAP-23) were localized to both the neurite and the inner-core cells in feline mesenteric PCs using immunocytochemistry. However, it has long been postulated that the first synapse of the PC occurs at the dorsal column nuclei, or spinal cord, and that no classical synapse is present in the distal ending of the capsulated receptor. While the historical interpretation of the function of glia has been that they provide support to the nervous system, recent evidence has shown that there is signaling occurring between glia and nerve cells. Thus, we believe that this glutamate-mediated synapse-like activity may play a modulatory role in the mechanotransductive process.

To obtain a secondary confirmation of this finding, RT-PCR testing is planned. Consensus sequences were developed based on the homologous mRNA for humans, rats, and mice and used to develop primers for PCR and RT-PCR on the feline PCs. Initial results confirm the functionality of the primers when using a DNA template. The primers are currently being optimized to determine ideal conditions that would increase the PCR yields before performing the RT-PCR.

The functionality of the primers suggests that the reactivity of the antibodies used in the ICC is accurate and not cross-reactivity due to the use of a consensus sequence for primer development and not the feline genome.

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Preface

One of the most challenging things about a thesis isn't the research, or choosing your project. The biggest challenge I faced was adapting techniques I had already learned to a new environment. One of the things I did not consider was learning new techniques for my thesis. My goal was to apply something I already knew and this was probably a mistake. Had I took on an entirely new project and learned new techniques I could have broadened myself to a greater extend than as I did applying my experience to a new field.

Molecular biology is something that I have spent years studying and doing research in, but it wasn't until I was in an academic environment that I became a serious challenge. The 'cushy' corporate world of funding and equipment spoiled my research habits and I was forced to adapt drastically. In my previous research, if supplies ran low there was a stockroom of nearly everything you could need for ANY type of experiment within a hundred feet of my lab. Working on this project, if I was missing something, my experiments fell days behind due to ordering through vendors, purchasing, and bureaucracy I genuinely wasn't used to. Aside form the inconveniences; I imagine it was something I wouldn't trade for the world because I will be a better researcher because of what I went through.

While it goes without saying don't procrastinate, your thesis is the one thing you really want to apply this to. An early start on your thesis can lend to graduate school and job interviews and will result in a better quality project.

Chapter 1: Pacinian Corpuscles (PCs)

PCs are the primary focus of this paper and thus a review of their structure and physiology is in order. While PCs are traditionally thought of as somatosensory mechanoreceptors they are also found in the mesentery of domestic cats, although there function is unknown in this location. It is hypothesized that PCs are functioning in the mesentery as baroreceptors (Spencer & Schaumburg, 1973; Tuttle & McCleary, 1975). PCs studied were harvested from the mesentery of cats and are of the same structure as those harvested from the human finger (Bell, Bolanowski, & Holmes, 1994).

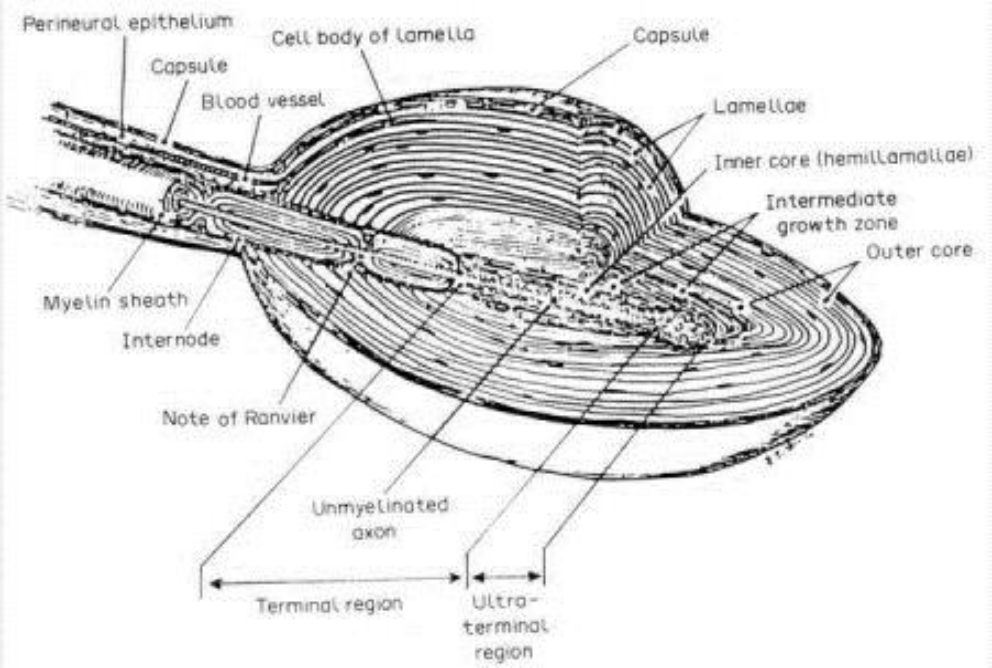
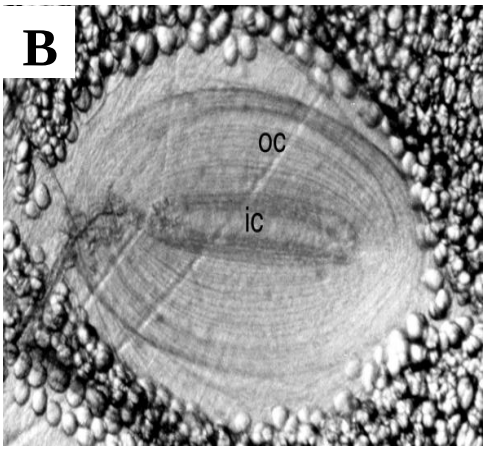


Figure 1.1: The fine anatomy of a PC. It is important to note the special relationships of the PC. This image was adapted from Bell *et. al* and is not drawn to scale (1994).

As seen in Figure 1.1, the structure of a PC is quite complex. The main components of a PC are a neurite in the center wrapped by layers of concentric lamellae called the capsule. The capsule is divided into two regions, the inner and

outer core and the two regions are separated by the intermediate growth zone or intermediate layer. The inner core is made up of very small and densely packed hemilamellae with collagen and fluid between the layers. The inner and outer core extracellular space actually contains collagen fibers that run longitudinally to the cells of the lamellae. One of the most notable features of the inner core is its bilateral symmetry. The lamellae are semiannular and thus also have been called hemilamellae.



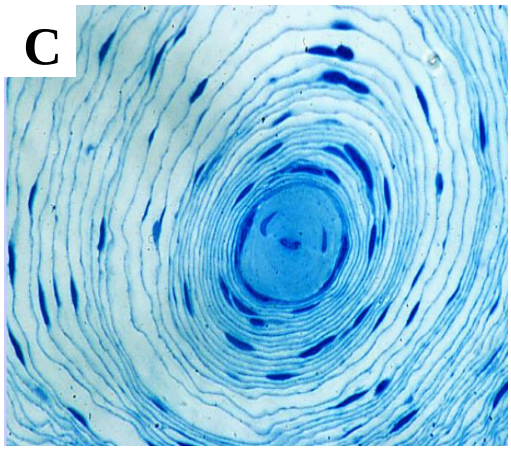


Figure 1.2 Light Microscopy of PCs.

A – The white ovals are PCs harvested from the mesentery of a cat. The golden globules in the background are fat.
B- A longitudinal section of a PC imaged with contrast microscopy to show the Inner Core (IC) and Outer Core (OC) layers.
C – A PC in transverse section stained to show the nuclei of the lamellar cells and the elliptical neurite in the center.

Images A & B Adapted from Bell *et. al.* (1994). Image C from Pawson *et. al* (2000).

It is believed these lamellae are modified Schwann cells. This is due to the fact that the inner core contains S-100, a calcium binding protein found in Schwann Cells. S-100 is localized exclusively to the inner core and not the outer

core or the neurite of the PC (Pawson, Slepecky, & Bolanowski, 2000; Vega et al., 1990; Zelená, 1994). The inner core also contains glial fibrillary associated protein (GFAP) (Pawson, Slepecky, & Bolanowski, 2000).

The inner-core cells are connected by gap junctions (Ide & Hayashi, 1987). This allows the cells to be electrically coupled. The gap junction connection may possibly allow calcium oscillations to travel along the lamellae in waves and has the potential to be part of a glial to glial signaling network. In addition, the inner core is very tightly layered and individual layers are almost indiscernible under light microscopy as seen in Figure 1.2B.

The outer core is made up of fibroblast cells in concentric layers around the intermediate membrane (Zelená, 1994). The outer core is a connected cell layer that is not hemilamellae like the inner core. The outer core also has much wider fluid and collagen filled space between the lamellae. The cells that make up the outer core are connected by tight junctions and this holds the extracellular fluid in place(Ide & Hayashi, 1987). The tight junctions also add to the mechanical stability of the outer core. Due to the tight junctions and intermediate layer, the extracellular fluid of the inner and outer cores is electrically isolated. The outer core holds its shape because the fluid layers are pressurized (Lowenstein, 1971).

The neurite that the innervates the PC has its cell body in the dorsal root ganglion and is composed of three distinct regions in the capsulated and most distal portion of the receptor. The three regions the capsule are: a terminal bulb or ultra-terminal region at the distal end of the PC, an unmyelinated middle region

in the inner core known as the terminal region, and a myelinated pre-terminal segment (Lowenstein, 1971; Zelená, 1994). As the neuron enters the PC it transitions from myelinated to unmyelinated. At this transitional zone of the inner core there is a hemi-node of Ranvier that separates the terminal and pre-terminal regions. The terminal region of the neuron is characterized by longitudinal neurofilaments and microtubules as well as a large number of mitochondria. Throughout the length of the terminal neurite there are projections into the clefts of the hemi-lamellae called filopodia (Lowenstein, 1971; Spencer & Schaumburg, 1973; Zelená, 1994). While filopodia lack mitochondria, at the base of each filopodia there is a collection of both mitochondria and clear core vesicles (Bolanowski, Schuyler, Sulitka, & Pietras, 1996). The clear core vesicles have been described in other somatosensory receptors (i.e. muscle spindles) as synaptic-like vesicles (SLVs) by Bewick *et. al*,(2005) and this term will be used to describe them. The filopodia also lack neurofilaments and microtubules, but they are rich in actin. The actin rich region could be important for mechanical signal transduction much like the inner hair cells of the ear (Pawson et al., 2000).

At the distal end of the neurite, the last 10-20% of the neurite is represented by the ultra-terminal region. This region has numerous filopodia which project into the outer-core region (Spencer & Schaumburg, 1973). The ultraterminus also contains numerous vesicles, lysosomes, and multivesicular bodies. The function of the vesicles has not been identified.

PCs respond primarily to high-frequency mechanical deformations of the receptor. They do not respond to uniform or constant stimuli, rather they respond to sudden changes in pressure due to mechanical deformation of the receptor and as such have been physiologically described as rapidly adapting mechanoreceptors. It is hypothesized that this is due to the mechanical properties of the lamellae around the nerve (Lowenstein, 1971).

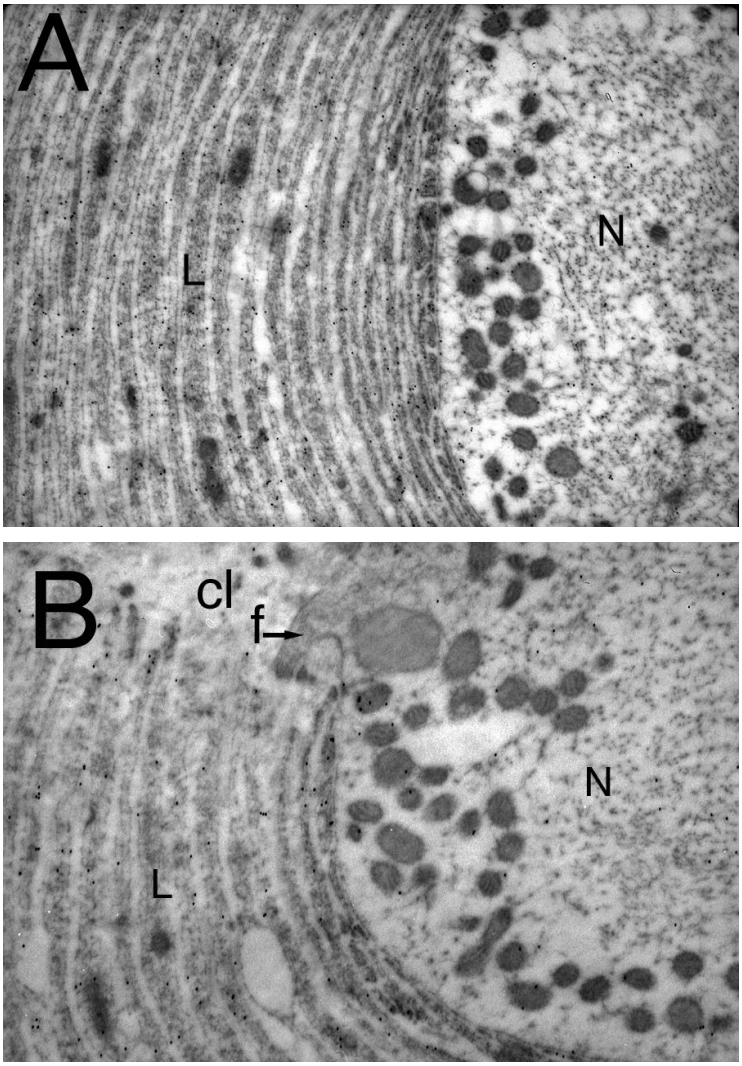


Figure 1.3 Electron micrographs of PC's terminal neurite labeled with anti-brain Type II voltage-gated sodium channel. A) Both the neurite (N) and its membrane show staining as well as the inner core lamellae (L). Calibration Bar = 626 nm. B) A closer view of the inner-core lamellae (L), the neurite (N) with a prominent filopodia (f) extending into the hemi-lamellar cleft (cl) which is a fluid-filled space containing collagen fibers. Calibration Bar = 584 nm. (Pawson & Bolanowski, 2002)

Previous Research

Recent work by Pawson and Bolanowski (2002) has shown the presence of voltage gated sodium channels on both the neurite and the lamellae of the PC. They have also

identified the presence of synaptic machinery using immunocytochemistry: glutamate, Vesicular Glutamate Transporter-1 (Vglut1), Metabatropic Glutamate Receptors, Vesicle Associated Membrane Protein-1 (Vamp1) also called synaptobrevin 1, and Synaptosomal-Associated Protein (Snap-23) (Pawson & Bolanowski, 2004). These proteins are components the SNARE signaling hypothesis in neurons. However, Araque *et.* al (2000) have found SNARE proteins resulting in the excocitotic release of glutamate from astrocytes, another type of glial cell. Additionally identification of clear-core vesicles (SLVs) in both the neurite and the lamella suggest that the synaptic machinery is present for

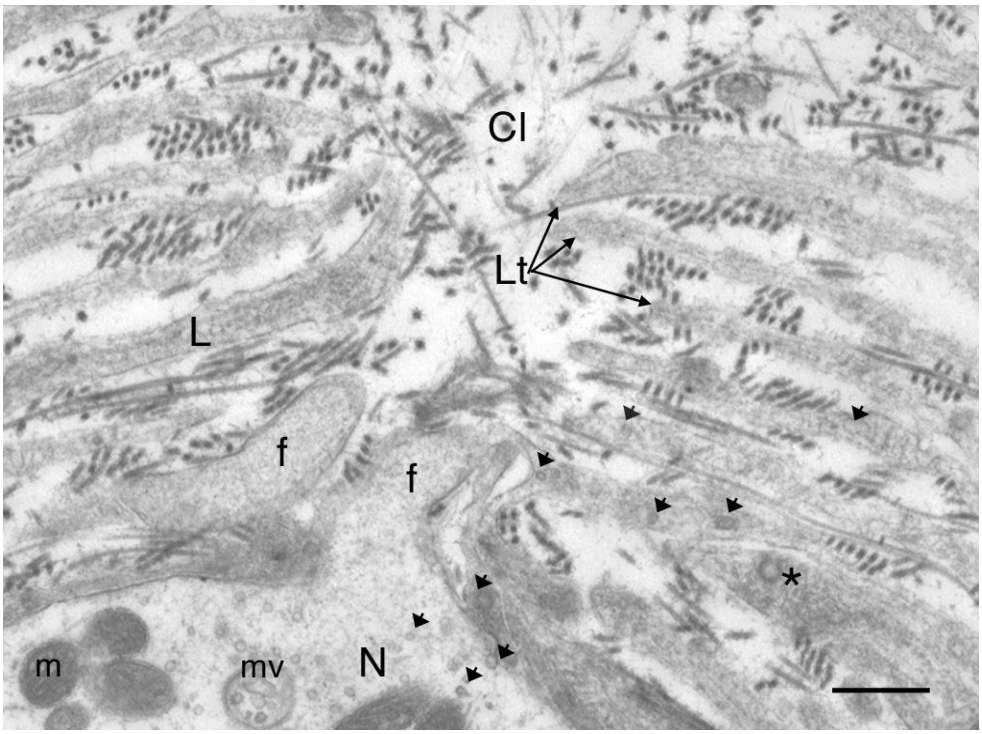


Figure 1.4 Electron micrographs of cross sections through isolated cat mesenteric PCs. A) Control micrograph showing the terminal neurite (N), with the filopodia (f) extending into the hemi-lamellar (L) cleft (Cl). Tips of the hemi-lamella (Lt) can be seen containing many vesicles (arrowheads). There are also vesicles (arrowheads) in the neurite portion as well as a multivesicular (mv) body. These are seen mostly at the base of the filopodia accompanied by clusters of mitochondria (m). Image from (Pawson & Bolanowski, 2004).

possible chemical interactions that have not been previously described.

This result was surprising because identifying the proteins on the modified Schwann cells suggests that they are possibly involved in the transmission of electrical activity involved in transduction. The current theory is that the channels and some components of synaptic machinery are present to modulate the receptivity of the PC. The unique design of the system and the possibility of glutamate containing SLVs, as described by Bewick *et.* al (2005), poses two interesting hypotheses. It is possible that the glutamate SLVs could be released from the neurite and stimulate the modified Schwann cells of the lamella or it possible that the lamella could be releasing glutamate to stimulate the neurite. Either possibility could result in changing the receptor potentials and result in modulation of the PC. The ability of the lamella to be electrically coupled to one another via gap junctions would result in rapid propagation of this message from one region of the lamellae to another.

It is the goal of my thesis to confirm these results using RT-PCR technology. A secondary confirmation is needed to back-up the immunocytochemistry data because some of the antibodies were not raised to be anti-cat. RT-PCR would confirm the specific reactivity of the antibodies used. RT-PCR is a process whereby the mRNA's (messenger transcript) for a gene are amplified using a PCR and reverse transcription in order to monitor gene expression and the manufacturing locations of a protein.

Chapter 2: Neuron-Glia Signaling

When thinking of the nervous system, one of the first images most people get is of nerves sending information throughout the body. Secondary thoughts are usually about the injuries from car accidents and athletics that damage the spinal cord and cause paralysis. What few people think of are the glial cells that are the second type of cell that make up the nervous system and outnumber nerves nine to one (Fields, 2004). Over the past half a century research into the nervous system has focused largely on the nerves themselves as they are known to be the information carrier and disregarded glial cells. Over the past decade there has been a slight shit in research focus to include glial cells into the scope of neuroscience and neurological research.

While the complete functions of glial cells are more or less unknown there is a growing body of research that suggests they might be a much larger player in the function of the nervous system than originally thought. For example, an analysis of the brain of one of the most world-renowned scientists of all time, Albert Einstein, showed that areas of the cerebral cortex that process higher neurological functions, exhibited nerve to glia ratios the were approximately half that of the average male (Diamond, Scheibel, Murphy, & Harvey, 1985). This information coupled with findings that glial cells influence formation of synapses that are essential to long term memory and learning (Fields, 2004). The finding suggests that the increased number of glial cells in Einstein's brain could correlate to his intelligence. While this was discovered in the mid 1980's, it took another decade for scientists to begin pinpointing the value of glia. Since initial findings

suggested that there may be reciprocal signaling between nerve cells and glia, the idea has begun to gain weight in the scientific community (Carmignoto, 2000).

One of the main reasons for the recent developments in glia-science (as opposed to neuroscience) has been technological innovations. Twenty years ago it wasn't possible to image calcium concentration changes or detect minute milli- or microvolt changes in a cell in the noisy background of a living environment. When scientists discovered that glia lacked the properties to carry action potentials along their membrane like a nerve cell, they were quickly discounted as important for electrical signaling and science moved on (Araque, Carmignoto, & Haydon, 2001; Fields, 2004).

The nervous system has two types of synapses or connections between adjacent or connecting cells in a nervous pathway. The first connection is a direct physical connection that allows a voltage to travel through the nervous system like electricity through a wire. The second type of synapse is a chemical synapse. This involves two adjacent cells coming into the near vicinity of each other but not touching. Specialized portions of the cell membrane of the presynaptic cell release vesicles containing neurotransmitters to receptors on the postsynaptic cell (Bear, Connors, & Paradiso, 2001).

While the traditional synapses are the standard in neuroscience, Synapse-like regions contain proteins or structures specific to the synapse. The reason they are considered synapse-like is due the traditional definition of a synapse being limited to a one way nerve to nerve connection.

Astrocyte Signaling

Astrocytes are a star shaped glial cell of the central nervous system that act as the blood-brain barrier to transfer metabolic wastes, oxygen, and nutrients between capillaries and neurons of the brain (Kandel, Schwartz, & Jessell, 1991). They have also been found to express neurotransmitter receptors and also release neurotransmitters via exocytosis. When astrocytes are activated via the neurotransmitters, they show large internal calcium oscillations (Zonta & Carmignoto, 2002). They also respond directly to current injections and as a result can trigger action potential generation in neighboring neurons(MacVicar, 1984). These calcium oscillations have become one of the common foundations of other methods of glial signaling and could possibly be found in PCs (Fields & Stevens, 2000; Zahs & Newman, 1997). The frequency of the calcium oscillations in astrocytes is dynamically linked to the neuron that initiated signaling of it and thus represents an active communication element (Carmignoto, 2000).

In addition to the signaling that has been discovered in astrocytes, there has also been a growing amount of research into their synaptic like activities. The exocytosis of neurotransmitters from SLVs exhibits a calcium dependent nature like that of the synaptic vesicles in neurons. Carmignoto (2000) also suggests that astrocytes have all of the components to represent a synapse meaning that they act as the presynaptic terminal to a postsynaptic neuron. Newman (2003b) goes far enough to say that synapses must be looked at as a three component system, the presynaptic neuron, postsynaptic neuron, and the glial cells that normally

ensheath them that have both presynaptic receptors and postsynaptic neurotransmitter release to the postsynaptic neuron. Both of these signaling hypotheses are novel ideas and need further research. It should also be noted that the details of this interaction haven't been defined.

Schwann Cell Signaling

Schwann cells are myelin secreting glial cells of the PNS that electrically insulate nerves to promote faster salutatory conduction (Bear et al., 2001). Schwann cells have been found to exhibit inhibitory properties via the release of ATP (Newman, 2003a). The majority of the work in other glial cells shows neurotransmitter release as an excitatory measure of signaling and this result is interesting for future research.

Chapter 3: Materials and Methods

Tissue Preparation: PCs and brain tissue were harvested from an anesthetized cat. The PCs were isolated individually from the mesentery of the cat's abdomen. The PCs were dissected out of the cat and trimmed of excess material to limit the sample to the outer core, inner core, and the neurite. PCs were then frozen in Krebb's solution in liquid nitrogen and stored at -80°C until mRNA was extracted. Brain tissue was cut from the cerebellum and also frozen in liquid nitrogen and stored at -80°C.

DNA Extraction: DNA was extracted using the Qiagen DNeasy system. Three 25 mg brain tissue samples were used for the extraction. The tissue was thawed and minced and the Qiagen protocol (Appendix 1) was followed. The protocol was modified to allow 24 hours for cell lysis.

mRNA Extraction: 7-10 PCs were used for mRNA extraction. They were minced and used in the Invitrogen Micro-Fast Track 2.0 mRNA Extraction Kit (Appendix 1). The protocol was followed without modification.

Primer Design: Primers were designed for PCR using Primer3 at <http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi>. The selection used regions found to be homologous between the human, rat, and mouse genomes using the mRNA transcript for Vglut1, Vamp1, and SNAP-23. Due to the inconsistencies in the rat sequence, the SNAP-23 alignment was performed using only the human and mouse sequences.

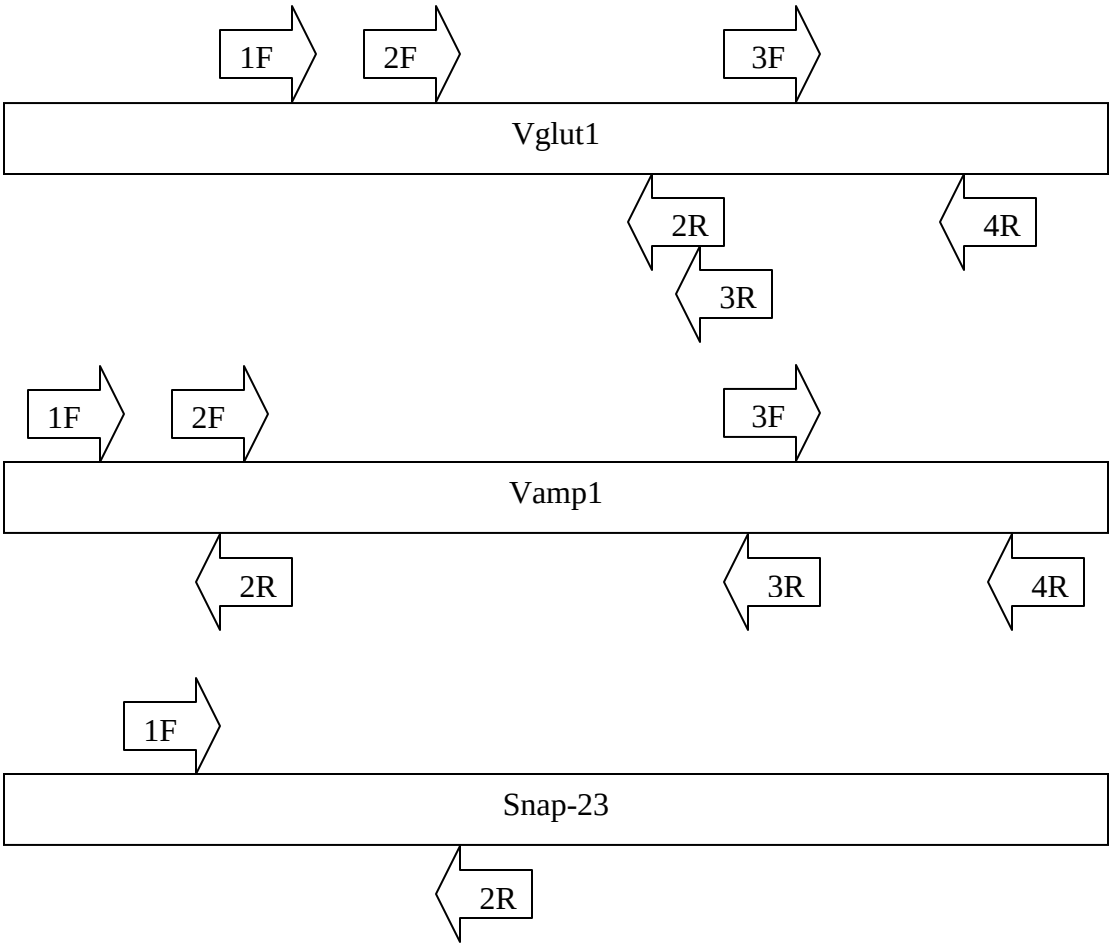


Figure 3.1 Primer alignments. The placement of each primer position with respect to the consensus mRNA sequence for the gene. The span of the primer is covered from the back end of arrow to the tip. There was an error in the design region for Vglut 2R and 3R as shown by their overlap above.

Once a region of high homology was found, that sequence was given to Primer 3 to design a primer, forward and reverse for the region. Due to the unknown factor in working with the feline genome more primers were designed in an effort to produce the best primer combinations possible. For Vglut1 and Vamp1, four regions of homology were used to generate a forward, reverse, and two internal primer pairs to yield six primers (three forward, and three reverse) for each gene. For SNAP-23 only one primer pair was designed due to sequence inconsistencies.

Optimal design conditions were 60°C melting temp and a desired length of approximately 20 bases. A feline beta actin primer was found and developed for use as a control (Rottman, Freeman, Tonkonogy, & Tompkins, 1995)

Primer Name	Primer Sequence (5' →3')
Vglut1F	GCTCCTTTTTCTGGGGCTAC
Vglut2F	ATGGCTGTGTCATCTTCGTG
Vglut2R	GAGTATCCGACCACCAGCAG
Vglut3F	GTGGCCATCTCCTTCCTG
Vglut3R	AGGACCAGGAAGGAGATGG
Vglut4R	TTCTCCTCGCTCATCTCCTC
Vamp1F	GACCTCCACTTCCTCTTTCAG
Vamp2F	GCTATCTGTGCCATCATCGTG
Vamp2R	CACGATGATGGCACAGATAGC
Vamp3F	ACCCCCTTCTGTGTCTCTGC
Vamp3R	AGACACAGAAGGGGGTCCAG
Vamp4R	TGTGGTAGGAATTTGTATTTTTCG
Snap1F	AACTAATGATGCCAGAGAAGATG
Snap2R	CCAAATCTTGAACGCAAAAG
Beta Actin F	ATGTGCAAGGCCGGCTTCG
Beta Actin R	TTAATGTCACGCACGAATTTCC

Table 3.1 Primer Sequences. The primer sequences that resulted from inputing the homologous regions to the Primer 3 utility online. The naming convention that was used contained the primer direction (F or R) and the region of homology. The genes of interest were abbreviated as follows: Vglut1-vglut, Vamp1-Vamp, and Snap-23-Snap. The region of homology is the number relating the to the region that the primer matches to. Therefore there is little or no overlap between a forward and reverse primer from the same region as seen in Figure 3.1.

DNA Quantification: Quantification of nucleic acids was performed using the Invitrogen DNA Dipstick system. The protocol was not altered (Appendix 1).

Gel Electrophoresis: Agarose gels were made using 1.5% electrophoresis grade agarose and 1X TAE. Ethidium Bromide (EthBr) staining was used to label the DNA and provide contrast under UV light conditions. Polaroid 667 film and an EthBR filter was used to image the gels. Gels were electrophoresed at 100V and run for as long was necessary for optimal separation.

Polymerase Chain Reaction (PCR): Invitrogen PCR Reagent system (cat# 10198-018) was used for PCR. The system was used following the methods

prescribed by Invitrogen and can be found in Appendix 1 for reference. Initial PCRs were performed with both 55°C and 60°C to try to determine an optimal annealing temperature. After primer fidelity was confirmed using PCR, the reactions were repeated for optimization using 50ul reactions at a temperature range from 50°C -60°C. Cycle steps and number of cycles are reported with results.

Gene	Primer Combination	mRNA Product (bp)
Vglut 1	1F-2R	830
Vglut 1	1F-3R	865
Vglut 1	1F-4R	1150
Vglut 1	2F-3R	725
Vglut 1	2F-4R	1010
Vglut 1	3F-4R	310
Vamp1	1F-2R	355
Vamp1	1F-3R	2200
Vamp1	1F-4R	2480
Vamp1	2F-3R	1870
Vamp1	2F-4R	2150
Vamp1	3F-4R	295
Snap-23	1F-2R	430
Beta Actin	1F-2R	590

Table 3.2. The anticipated products from RT-PCR or PCR using a cDNA template. Each primer combination is shown with the expected product from an RT-PCR assay. The expected products in a PCR using a DNA template would be larger if it included introns.

Results

DNA extraction: The DNA extraction yielded sufficient quantity of nucleic acids. The final yield was roughly 600ul of fluid containing approximately 3ng/ul concentration based on the DNA dipstick test. It was found that 50ul PCR reactions are efficient and produce a large yield of product with 3ul of DNA.

PCR: The results from the PCR were somewhat troublesome in the difficulty to repeat themselves. A great deal of time was spent developing confidence in the instrument with controls. Using the initial suggestions from the manufacturer settings for cycle length of 30 seconds for denaturing, 45 seconds for annealing, and 1.5 minutes for extension with 30 seconds and 25 cycles, results were found to be sporadic (results not shown). Cycle length and number of cycles were extended to double the cycle length and 30 cycles. It was found that the reactions required double the recommended times from the Invitrogen recommendations to 1 minute denaturing, 1.5 minutes annealing, 3 minutes extending for 30 cycles. A silicon oil overlay was also found to be required for any PCR product to be formed.

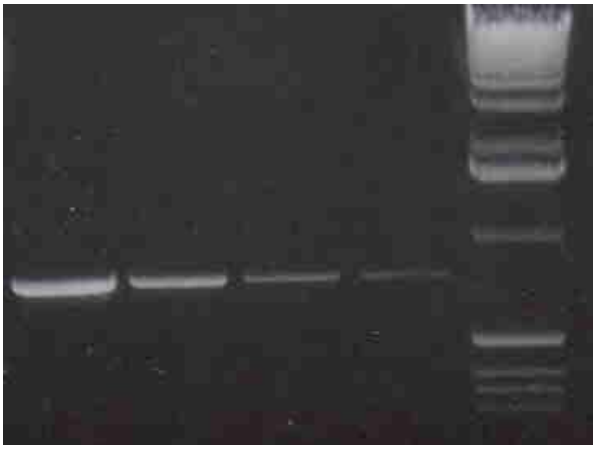


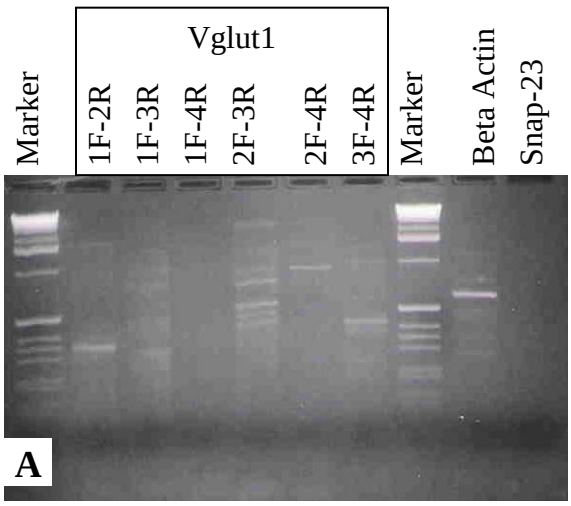
Figure 4.1 Agarose gel showing Control PCR. The final control PCR is shown above. The right lane is a DNA marker and the other four lanes from left to right are serial dilutions: 1, 1:1, 1:10, 1:100.

PCR reactions performed using a feline DNA template and the designed

primers were performed at both 55 and 60 Figure 2. Due to the lack of a single high quality reaction, primers were further optimized from 50 to 60 in 2 degree increments. Results of these tests are still pending.

Overall the primers that were designed exhibited good reactivity with the feline genome. All of the primers with the exception of one pairing exhibited some reactivity which is an encouraging results with the design performed for an unpublished genome.

Before primer optimization is complete, the preliminary reaction indicates ideal primer pairs are Vglut 1F-2R and 2F-4R as shown by the single bands in Figure 4.2. Additionally Vamp2F-4R can be eliminated due to the product size being too small for the mRNA product.



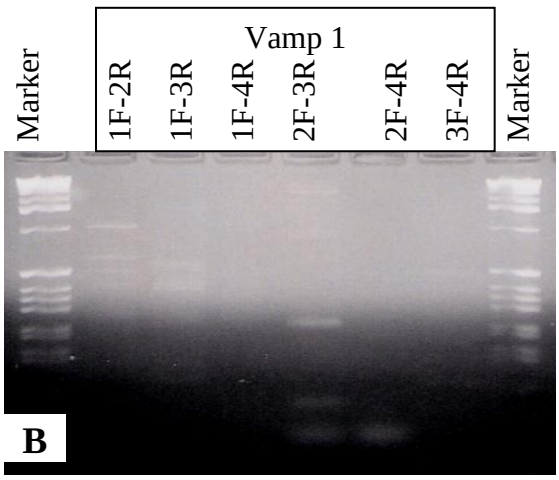


Figure 4.2 55°C Annealing PCR. Both Part A and B show the same PCR reaction on different rows of an agarose gel. Part B only shows Vamp1 primers, while A shows Vglut1, Beta Actin, and Snap-23.

mRNA Extraction and cDNA synthesis: The RNA extraction was performed

using 10 PCs to harvest the RNA from. RNA was not quantified due to the expectedly low yield of material. Immediately upon extracting the mRNA, cDNA synthesis was performed to provide better stability for storage and future analysis. A double synthesis was used in order to increase the cDNA yield as much as possible. Like the mRNA, the cDNA was not quantified due to low expected yields. cDNA is ready for PCR pending the outcome of primer optimization.

Discussion

While this experiment is still very much a work in progress, a great deal has been learned along the way. As the experiment moves forward into primer optimization, I am confident that the primers that will be developed will prove effective given the time to optimize them to a specific temperature.

One of the theories that cannot be ignored is the possibility of the neurite in the PC releasing SLVs. Bewick *et. al* (2005) identified and recorded glutamate containing SLV activity in the muscle spindle and determined it was linked to mechanotransduction. The SLV signaling they observed was directly correlated to muscle spindle stretch. Researchers did note calcium dependence with their activity which seems highly consistent with other glial signaling method. This work has been furthered additionally by immunocytochemistry data showing intense labeling for Vglut1 in muscle spindle afferents (Wu et al., 2004). The suggestion that the glutamate activity present in the spindles could function similarly to these found in the PC cannot be ignored.

Bewick (2005) formed the "Autogenic Modulation" hypothesis on glutamate SLV activity shown in Figure 5.1. The study showed glutamate exocytosis and glutamate reactivity was self-stimulatory. However, his work did completely ignore the capsule surrounding the muscle spindle. Due to anatomical and physiological data already collected on the PC and the presence of clear cored SLVs, that may contain glutamate, in both the neurite and the lamellar cells, a secondary hypothesis must be developed. The "Reciprocal Signaling" hypothesis, Figure 5.2, contends that neurotransmitters could be released from either the

lamellar cells or the neurite to signal each other, themselves, or both. This is the hypothesis that I believe to be most fitting for the PC due to the proximity of the SLV containing modified Schawnn cells of the inner-core.

The modulation that occurs in either hypothesis could be something as simple as amplifying the output signal or as complicated as changing the frequency response or adaptation process. This is still unknown at the moment and an intense focus of future research.

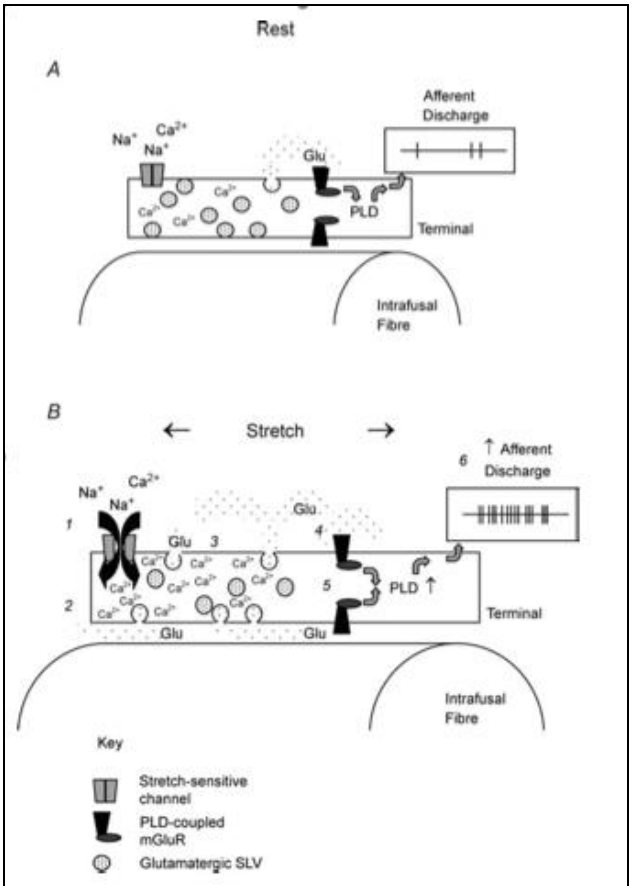


Figure 5.1 The Autogenic Modulation Hypothesis. The resting state (A) and stretched state (B) of a muscle spindle is shown with the resulting calcium wave and glutamate exocytosis. Image from (Bewick, Reid, Richardson, & Banks, 2005).

Further studies will use the optimized primers to actually perform the RT-PCR. If techniques could be developed to separate the inner-core, outer-core, and neurite, differential RT-PCR data would show specific expression of genes of interest in each component of the PC. This could also be performed quantitatively to measure the amount of mRNA present which correlates to protein expression levels.

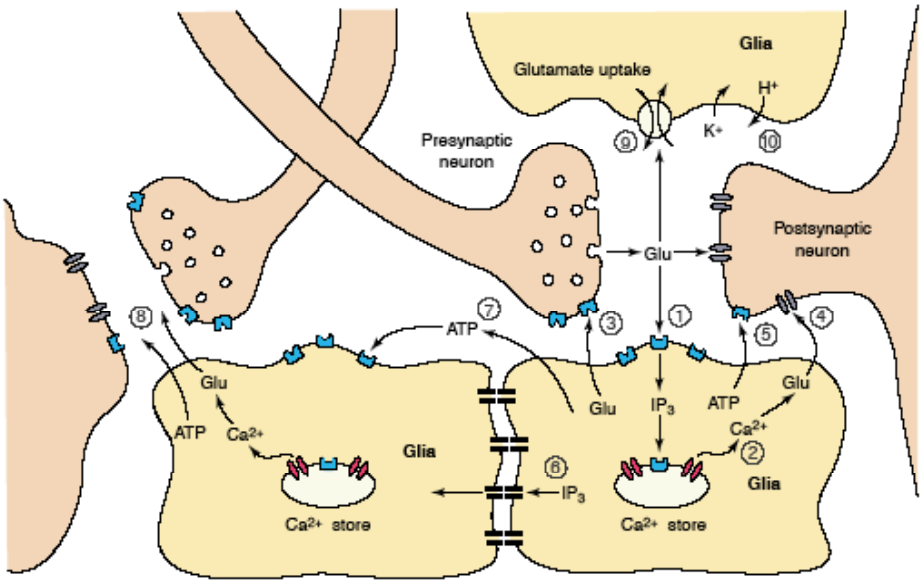


Figure 5.2 The Reciprocal Modulation Hypothesis. Reciprocal modulation is shown in astrocytes. Release of glutamate (Glu) from the presynaptic terminal activates glial receptors (1), evoking an increase in Ca2þ levels (2) and the release of glutamate from glia. Glutamate activation of presynaptic receptors (3) regulates transmitter release, while activation of postsynaptic receptors (4) directly depolarizes neurons. Stimulation of glia also elicits the release of ATP, which inhibits postsynaptic neurons by activating A1 receptors (5). Activation of glia might also evoke an intercellular Ca2þ wave, which propagates between glia by diffusion of inositol (1,4,5)-trisphosphate (IP3) through gap junctions (6) and by release of ATP (7), and results in the modulation of distant synapses (8). Glia also modulate synaptic transmission by uptake of glutamate (9) and by regulating extracellular Kþ and Hþ levels (10). Other glial–neuronal interactions are not illustrated. These include glial activation by neurotransmitters other than glutamate and glial regulation of NMDA-receptor-containing synapses by release of D-serine.

While the image is accurate to the model, there are small modifications necessary to apply it to the PC. The glia at the bottom are shown connected via gap junctions and that is true of the inner core lammela that could conduct an electrical signal from one end of the PC to the other. The modification to apply this to PCs is that there is no traditional synapse and no postsynaptic neuron in the capsule. Image and legend from (Newman, 2003b)

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Appendix 1

Table of Contents **Page**

1) DNA Extraction Protocol – Qiagen DNeasy Kit

Qiagen DNeasy Tissue Handbook Page 18-20, 03/2004

Protocol: Purification of Total DNA from Animal Tissues

Important points before starting

■ Before using the DNeasy Tissue Kit for the first time, read "Important Notes" on
pages 10–17.
■ If using fixed tissue, please refer to Appendices B and C (starting on page 30).
■ All centrifugation steps are carried out at room temperature (15–25°C).
■ Vortexing should be performed by pulse vortexing for 5–10 s.
■ Optionally, RNase A may be used to digest RNA during the procedure. RNase A
is not provided in the DNeasy Tissue Kit (see "Copurification of RNA", page 14).

Things to do before starting

■ Buffers ATL and AL* may form precipitates upon storage. If a precipitate has formed in either buffer, incubate the buffer at 55°C until the precipitate has fully dissolved.
■ Buffers AW1* and AW2† are supplied as concentrates. Before using for the first
time, add the appropriate amounts of ethanol (96–100%) to Buffers AW1 and AW2 as indicated on the bottles.
■ Prepare a 55°C shaking water bath for use in step 2 and a 70°C water bath or heating block for use in step 3.
■ If using frozen material, equilibrate the sample to room temperature.

Procedure

1. Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, place in a 1.5 ml microcentrifuge tube, and add 180 µl Buffer ATL.

Ensure the correct amount of starting material is used (see page 10). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used. It is advisable to cut the tissue into small pieces to enable more efficient lysis.

*Contains chaotropic salt. Not compatible with disinfecting agents containing bleach. For safety information,
see page 7.
† Contains sodium azide as a preservative.

2. Add 20 µl proteinase K, mix by vortexing, and incubate at 55°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely. After incubation the lysate may appear viscous, but

should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, please see the "Troubleshooting Guide" on page 26 for recommendations.

Optional: RNase treatment of the sample. Add 4 µl of RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature (15–20°C). Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. If RNA-free genomic DNA is required, carry out this optional step. If residual RNA is not a concern, omit this step and continue with step 3.

3. Vortex for 15 s. Add 200 µl Buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70°C for 10 min.
It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during the incubation at 70°C. The precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL. In this case, vigorously shaking or vortexing the preparation before addition of ethanol in step 4 is recommended.

4. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column.

5. Pipet the mixture from step 4 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.*

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 7 for safety information.

7. Place the DNeasy Mini spin column in a 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
This centrifugation step ensures that no residual ethanol is carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol

occurs, empty the collection tube and reuse it in another centrifugation step for 1 min at 20,000 x *g* (14,000 rpm).

8. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.
Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 14).

9. Repeat elution once as described in step 8.
A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 8 can be reused for the second elution step.

Note: More than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

2) Invitrogen FastTrack® 2.0 Kit Version J , p7-12, 8/2001

Preparation of Samples.Fresh and Frozen Tissue

Preparation of Tissue for Storage

To achieve high yields of mRNA from plant, insect, or mammalian tissues, ~0.5-1 gram of tissue per isolation is recommended. All lysis and washing steps must be performed to ensure consistently high yields. Please see page 8 for plant tissue preparation.

1. Quickly excise the tissue, cut into pieces of 1-2 cm2 with a sterile knife or razor blade, and immediately freeze in liquid nitrogen.
2. Store the tissue at -70°C in a polypropylene tube with a screw cap. Frozen tissue is stable at -70°C for over 1 year. Proceed to Preparation of Fresh or Frozen Tissue, below.

Preparation of Fresh or Frozen Tissue

It is important that frozen tissue is thawed and homogenized in the presence of the lysis buffer. This will ensure immediate inactivation of any RNases that are released as the cells lyse. Complete homogenization is critical for complete cell lysis and inactivation of RNases.

Be sure to clean and wash the homogenizer tip, then autoclave and bake for 3 hours or overnight at 210°C.

1. Place ~1 g fresh or frozen tissue in a tared (pre-weighed), sterile, 50 ml centrifuge tube and weigh. Add 15 ml FastTrack® 2.0 Lysis Buffer (page 5).
2. Quickly homogenize the tissue using a motor-driven homogenizer such as Tissuemizer (Teckmar) or Omni Mixer Homogenizer (Omni International). Start at a low speed and slowly increase speed until a smooth homogenate with no visible particulate matter is obtained (~15-30 seconds). Keep foaming to a minimum by adjusting the speed.
3. Proceed to the basic mRNA isolation procedure, page 9, Step 1.

Alternative Procedure

If a motor-driven homogenizer is not available, tissues may be homogenized using a mortar and pestle, and a Dounce homogenizer. Be sure to clean the equipment, then autoclave and bake before use.

Weigh out ~1 g of fresh or frozen tissue and place into a mortar precooled with liquid nitrogen. Cover the tissue with more liquid nitrogen and grind with a pestle until a fine powder is obtained. Add more liquid nitrogen as needed to keep the tissue covered with liquid nitrogen while grinding.

2. Transfer the suspension of liquid nitrogen and crushed tissue into a sterile 50 ml centrifuge tube containing 15 ml of FastTrack® 2.0 Lysis Buffer (page 5).
3. Transfer to a sterile Dounce homogenizer at room temperature. Homogenize with 10-12 strokes. Proceed to the basic mRNA isolation procedure, page 9, Step 1.

Isolation of mRNA

Incubate the cell lysate produced in the sample prep procedures (pages 6.8) at 45°C for 15-60 minutes. If insoluble material persists, centrifuge at 4,000 x g for 5

minutes at room temperature and transfer the supernatant to a new tube.

Incubation is important for complete digestion of proteins and ribonucleases. A 60 minute incubation is recommended for tissues, while cell material is effectively digested with a 15 minute incubation. You may wish to optimize the time of incubation for your particular sample.

2. Adjust the NaCl concentration of the lysate to 0.5 M final concentration by adding 950 µl of the 5 M NaCl stock solution to each 15 ml lysate. Mix thoroughly by inversion.
3. Shear any remaining DNA by passing the lysate 3 to 4 times through a sterile plastic syringe fitted with an 18-21 gauge needle. This will yield a cleaner mRNA preparation. If solution is viscous, please see Troubleshooting on page 13.
4. Remove a vial of oligo(dT) cellulose from the dessicator and add the contents of the vial to the lysate.
5. Seal the tube and allow the oligo(dT) cellulose to swell for 2 minutes. The oligo(dT) cellulose should disperse readily.
6. Rock the tube gently at room temperature for 15 to 60 minutes.

   Rocking or rotating increases the efficiency of mRNA binding to oligo(dT) cellulose.
7. Pellet the oligo(dT) cellulose at 3,000 x g in a table-top or similar centrifuge for 5 minutes at room temperature. Remove the supernatant carefully from the resin bed. Proceed to the **Washing Procedure** below.

Continued on next page

Basic mRNA Isolation Method, Continued

Washing Oligo(dT) Cellulose

Gently resuspend the oligo(dT) cellulose in 20 ml of Binding Buffer. Centrifuge at 3,000 x g in a table-top or similar centrifuge for 5 minutes at room temperature. Remove the Binding Buffer from the resin bed.

2. Gently resuspend the resin in 10 ml Binding Buffer and centrifuge as in Step 1. Remove the buffer from the resin bed.
3. Gently resuspend the resin in 10 ml Low Salt Wash Buffer and centrifuge as in Step 1. This low salt wash will remove SDS and contaminating RNAs such as rRNAs.
4. Repeat Step 3 until the buffer no longer has bubbles in it after centrifugation (at least 2.4 times). After the last wash, resuspend the oligo(dT) cellulose in Low Salt Wash Buffer at a final volume of 800 µl.
5. Transfer the oligo(dT) cellulose into a spin-column (inside the spin-column/micro-centrifuge set) to perform the last washing steps. Centrifuge at 5,000 x g for 10 seconds at room temperature.

6. Remove the column from microcentrifuge tube and decant the liquid inside the tube. Repeat Steps 5 and 6 as many times as necessary (~2-3 times) to transfer the oligo(dT) to the spin column.
7. To wash, place the spin-column back into the tube, fill it to the top with Low Salt Wash Buffer (~500 µl) and mix the buffer into the cellulose bed with a sterile pipette tip. Centrifuge for 10 seconds.

Be careful not to damage the membrane as you will lose the resin (and your sample).

8. Repeat Step 7 at least 3 times or until the OD_{260} of the .flow-through. is < 0.05. Be sure to mix the buffer into the cellulose bed.

Basic mRNA Isolation Method, Continued

Elution and Precipitation of the mRNA

Place the spin-column into a new (sterile and RNase-free) microcentrifuge tube provided in the kit.

Add 200 µl of Elution Buffer and mix the buffer into the cellulose bed with a sterile pipette tip.

3. Centrifuge for 30 seconds, but **DO NOT** decant the liquid. The mRNA is now in the eluate.
4. Add a second 200 µl of Elution Buffer to the column, mix into the cellulose, and centrifuge again for 30 seconds.

   Steps 2 through 4 will elute the mRNA into the microcentrifuge tube.
5. Remove the column from the tube. The tube should now contain 400 µl. Do Not Discard. This is your mRNA sample.
6. Precipitate the mRNA with 0.15 volume (~60 µl) of 2 M sodium acetate (supplied) and 2.5 volume (1 ml) of 200 proof (100%) ethanol. Freeze on dry ice until solid.
7. Thaw and centrifuge in a microcentrifuge at maximum speed (16,000 x g) for 15 minutes at +4°C.
8. Remove the ethanol. Centrifuge briefly and remove traces of ethanol. Resuspend the RNA pellet in 20-50 µl of Elution Buffer (10 mM Tris, pH 7.5).
9. Determine the concentration of the mRNA (see below). The mRNA may be used immediately or stored indefinitely at -70°C.

Determination of RNA Yield

To determine the concentration of the resuspended RNA, dilute the sample 100 fold by adding 4 µl of sample to 396 µl of Elution Buffer. Use Elution Buffer to blank the spectrophotometer at 260 nm. Place the diluted sample into a 500 µl quartz cuvette and read the absorbance at 260 nm. Determine the RNA concentration by using the following formula:

$$[RNA] = (A_{260})\ (0.04\ \mu g/\mu l)\ D$$

D is the dilution factor (D = 100 in the above example). Determine the RNA yield by multiplying the concentration by the volume of the RNA. Note that the A_{260} must be > 0.05 to give an accurate RNA concentration.

FastTrack® 2.0 Quick Reference Card

If you routinely use the FastTrack® 2.0 mRNA Isolation Kit, you may wish to use our laminated Quick Reference Card as a checklist. Each step can be conveniently marked to keep track of centrifugations, transfers, and washes. Use a Sharpie® pen to check off items and wipe off with ethanol to re-use.

3) Invitrogen DNA Dipstick™ Kit Version C, p5-7, 8/2001

Using the DNA DipStick. Kit, Continued

Applying Sample

1. Make appropriate dilutions of the sample to be tested in sterile water or TE (bring the final concentration between 0.1 and 10 ng/⌠l). For unknown sample concentrations, try the undiluted sample along with dilutions of 1:10 and 1:100

2. If you are using the control DNA included in the kit, prepare dilutions of the control DNA as described on the previous page.

3. Avoiding contact with the membrane portion of the stick, place one DNA DipStick. per sample (membrane up) on a clean surface (the sample and its two dilutions can all be applied to the same DNA DipStick.).

4. Apply 1 ⌠l each of the sample directly on to the membrane without overlapping the spots.

5. Allow the spots to air dry for 5-10 minutes. You can place the DNA DipStick. under a light source for a few minutes to speed up the drying process. **Note**: Consistency is important when applying spots. Avoid contact between the membrane and the pipette tip. The sample may remain as a tight drop for a few seconds after application and then absorb into the membrane, forming an irregularly shaped spot. This will not affect the result of the assay in any way. If adjacent samples fuse together at this stage, the final result will be a round, blue-colored dot.

6. While the sample spots are drying, set up the solutions to develop the assay. To open the Developer, press upward firmly on the red cap.

Using the DNA DipStick. Kit, Continued

Procedure

1. To the labeled cuvettes from page 3, add □ 1 ml of Wash Solution in Cuvette #1 □ 1 ml of Coupling Solution in Cuvette #2 □ 1 ml of Developer Stock plus 1 drop of Developer into Cuvette #3. Cap Cuvette #3 and mix the Developing Solution by inverting.

2. Once the sample spots have dried, place the DNA DipStick. in Cuvette #1 (containing Wash Solution) for 10 seconds.

3. Transfer the DNA DipStick. into Cuvette #2 (containing Coupling Solution) and let it stand for 3 minutes. Place the DNA DipStick. vertically into the solution with the back of the DNA DipStick. against the cuvette wall to allow complete contact between the membrane and the Coupling Solution (see figure below). \

4. Remove the DNA DipStick. and rinse it with deionized water for 20 seconds, and place it back into the Wash Solution in Cuvette #1 for 4 minutes. The DNA DipStick. must be vertical with the back of the stick against the cuvette wall.

5. Place the DNA DipStick. into Cuvette #3 (containing the Developing Solution) for 2 minutes. The DNA DipStick. must be placed as vertically as possible (see figure above).
Continued on next page 7

6. Gently rinse the DNA DipStick. in the Wash Solution in Cuvette #1. After 20 seconds remove the DNA DipStick.. Place it flat (with membrane side up) to air dry.

7. After drying the DNA DipStick., compare the color intensities of the sample spots on the membrane to the control DNA or the standard chart on the DNA DipStick. Quick Reference Card to estimate the concentrations of the samples.

8. Dispose of the Coupling Solution in Cuvette #2 and the Developing Solution in Cuvette #3 in accordance with local and state waste disposal regulations. Rinse all three cuvettes with distilled water and re-use the cuvettes for future assays.

 The dots on the standard chart in the Quick Reference Card range in concentration from 0.1 ng/∫l to 10 ng/∫l and are applicable to single- and double-stranded DNA, RNA, and oligonucleotides.

 When comparing the sample dots to the standards, at least one of the sample concentrations should fall within the range of the standards,

 If the sample dots will are of intermediate intensity to those on the standard chart, then estimate a concentration value, or to achieve an optimal color match, prepare additional dilutions based on the initial result and repeat the assay.

 If the amount of the sample applied to the DNA DipStick. ranges from 10-500 ng/∫l, the intensity of the dot will not correlate to the amount of nucleic acid in the sample.

 If more than 500 ng/∫l of nucleic acid is applied, the

4) Invitrogen PCR Reagent System, **Cat. No.: 10198-018**, 1/2002

Basic Protocol:
The following protocol is optimized for the control DNA and the primers provided with this kit. This protocol may serve as a starting point for any PCR amplification. Critical parameters to optimize include incubation times and temperatures, concentration of *Taq* DNA Polymerase, primers, MgCl2, and template DNA (2). Since PCR is a powerful technique capable of amplifying trace amounts of DNA, all appropriate precautions should be taken to avoid crosscontamination. Amplification reactions should be assembled in a DNA-free environment. Use of "clean" dedicated automatic pipettors and aerosol resistant barrier tips are recommended.
Always keep the control DNA and other templates to be amplified isolated from the other components. PCR products should be analyzed in an area separate from the reaction assembly area.

1. Add the following components to a sterile 0.5-ml microcentrifuge tube on ice:

Components Volume	(µl)	Final Concentration
Autoclaved distilled water	78.5	
10X PCR buffer minus Mg*****	10.0	1X
50 mM MgCl2 *****	3.0	1.5 mM
10 mM dNTP mixture	2 0.	2 mM each
Primer mix (10 µM each)	5.0	0.5 µM each
Control DNA (200 ng/µl)	1.0	200 ng
Taq DNA Polymerase (5 U/µl)	0.5	2.5 units
Total Volume	100 µl	

If desired, a master mix of buffer, MgCl2, dNTP's and *Taq* DNA polymerase can be prepared for multiple reactions. This minimizes reagent loss and enables accurate pipetting.

*Note: 10X PCR Buffer plus Mg can be used instead of the separate 10X PCR buffer minus Mg and 50 mM MgCl2 to achieve the same final concentration.

2. Mix the contents of the tube and overlay with two drops of silicone oil.

3. Cap the tubes and centrifuge briefly to collect the contents to the bottom of the tube.

4. Incubate the tubes in a thermocycler at 94°C for 3 min to completely denature the template.

5. Perform 35 cycles of PCR amplification at:

Denature: 94°C for 45 s
Anneal: 55°C for 30 s
Extend: 72°C for 1 min 30 s

6. Incubate for an additional 10 min at 72°C and maintain the reaction at 4°C. The samples can be stored at -20°C until use.

7. Analyze the amplification products by agarose gel electrophoresis and visualize by ethidium bromide staining.